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14. ABSTRACT

XIAP is an exciting potential target for breast cancer therapeutics, but the specific functions of XIAP that contribute to breast cancer development or progression remain unknown. This project seeks to identify the properties of XIAP that play a crucial role in breast cancer, using mutated XIAP molecules that are deficient in specific cellular functions in both in vitro and in vivo models of breast cancer. The tasks of the first phase of this project have been completed: establishing breast cancer cell lines deficient in XIAP or express only mutant forms of XIAP that can be used in models of anchorage-independent growth or tumor formation in immunocompromised mice. In the next phase of this project, these cell lines will be evaluated to identify the role of XIAP in tumorigenesis, which will be a valuable guide in the development of therapeutics that specifically target tumor-related functions of XIAP and in the advancement of our understanding of breast cancer biology.

15. SUBJECT TERMS
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Introduction

Pre-clinical evidence suggests that X-linked Inhibitor of Apoptosis (XIAP) is an exciting potential target for breast cancer therapeutics, which provides strong motivation for the role of XIAP in breast cancer to be fully elucidated. XIAP is involved in multiple cellular functions that have been implicated in breast cancer. This project seeks to identify the properties of XIAP that play a crucial role in breast cancer, by examining mutated XIAP molecules that are deficient in specific cellular functions in both in vitro and in vivo models of breast cancer.

Body

The initial task of this project entailed developing in vitro and in vivo models of breast cancer cell growth. The models I was interested in developing are a xenograft model of cancer cell growth in immunocompromised mice and an in vitro model of anchorage-independent cell growth. As stated in the original proposal, the breast cancer cell lines our laboratory possessed at the time did not demonstrate tumor formation in vivo. I have now obtained cancer cell lines that robustly form tumors in immunocompromised mice from Marc Lippman's laboratory (University of Michigan). In my hands, these cell lines demonstrate anchorage-independent growth in soft agar. Based on these data from the Lippman laboratory and my results, I expect that these cell lines (principally the MDA-MB-231 line) will provide suitable models of breast cancer growth and progression. I have utilized these cell lines in the subsequent tasks of this project.

Using the cell lines identified in the first task, I proceeded to develop cell lines that are deficient in specific functions of XIAP. First, I developed cell lines that are stably deficient in XIAP expression using RNA interference (RNAi). Short hairpin RNAi sequences (shRNA) were stably integrated into the genome using a lentiviral vector. I used lentiviral vectors containing shRNA targeting XIAP or control sequences that out laboratory has previously developed, and I also optimized additional vectors targeting XIAP and control sequences. The effect of introducing the shRNA vectors on XIAP protein expression was examined by quantitative Western blot. In all cells infected with XIAP shRNA constructs, there was greater than 90% reduction in expression of XIAP at the protein level. In some cases, XIAP expression was regained after several passages in cell culture, suggesting that a population of uninfected cells is present. To address this problem in certain cell lines, cells were selected by fluorescence-activated cell sorting (FACS) for expression of a fluorescent marker (GFP or dsRed2) at an early passage after viral infection. Following cell sorting, loss of XIAP protein expression persisted through extended numbers of passage in vitro. In some cases, cell sorting was not necessary because the cell population was homogenously positive for the fluorescent marker, as determined by FACS.

To develop cell lines that are deficient in specific functions of XIAP, in addition to the lines that are completely deficient in XIAP expression, I utilized expression constructs of XIAP with point mutations that abrogate specific functions of XIAP (D148A, W310A, H467A).. These expression constructs also contain mutations that permit resistance to the RNAi constructs and were stably introduced into the cell lines using a similar lentiviral vector. An infected population of cell was selected by hygromycin resistance. I confirmed restored expression of XIAP protein in the cell lines by Western blot. In the cases of the D148A and W310A mutations and restored expression of wild type XIAP, expression of XIAP in these cell lines exceeded expression of endogenous XIAP in the parental cell line. Despite the difference in expression levels compared to the parental cell line, restored expression of wild-type XIAP in the deficient cell line is available as a control to account for the differences in expression levels. In the case of the H467A mutation, expression of this XIAP mutant was not a strong as other mutated XIAP constructs. Because transient expression of the mutant occurs at wild-type levels, this observation might lead to interesting new areas of research. However, in the scope of this project, the expression levels of the mutant XIAP cell lines will need to be carefully considered when interpreting experimental results.

Using the cell lines that are deficient in specific functions of XIAP, I set out to confirm that these mutations affect the ability of the cells to resist programmed cell death in a manner consistent with published results. XIAP is a known inhibitor of apoptosis, and loss of XIAP is reported to sensitize cancer cell lines to apoptosis. I treated cells with stimuli for both the intrinsic (ultraviolet irradiation) and extrinsic (TRAIL) apoptotic pathways in increasing doses. Surprisingly, for either stimulus the response of the XIAP-deficient cell lines was identical to cells with wild-type XIAP. After extensive investigation of these cell lines, our laboratory has determined that the loss of XIAP does not affect the proportion of cells that undergo cell death immediately following TRAIL stimulus, but rather it affects the number of cells that recover and proliferate over an extended amount of time. Based on this finding, I plan to re-evaluate the cell lines deficient in specific XIAP functions under these new conditions.

I have also examined our XIAP-deficient cell lines in the anchorage-independent growth model. Cells were suspended in soft agar in tissue culture plates, and the media was refreshed as needed. Over the next four to six weeks, cell growth was monitored by light microscopy and fluorescence. Colony formation was counted using ImageJ software (NIH, Bethesda, MD). In this model, the XIAP-deficient cells formed colonies with similar total area as wild-type cells. This observation suggests that XIAP does not contribute to the anchorage-independent growth of breast cancer cell lines. In combination with the results from studies of apoptosis, these findings indicate that the role for XIAP in breast cancer cell lines is sensitive to the type of apoptotic stimulus.

Key Research Accomplishments

- Identified breast cancer cell lines suitable for the intended in vitro and in vivo breast cancer models.
- Established breast cancer cell lines that are deficient XIAP.
- Developed breast cancer cell lines that are deficient in specific functions of XIAP by reintroducing XIAP mutants.
- Assessed the effect of loss of XIAP on sensitivity to apoptosis using extrinsic and intrinsic apoptotic stimuli.
- Assessed the effect of XIAP on anchorage-independent growth.

Reportable Outcomes

- Presented results at the University of Michigan Medical Scientist Training Program annual retreat.
- Presented results at the University of Michigan Department of Pathology annual research symposium.
- Presented results at the University of Michigan Department of Pathology seminar series.

Conclusion

XIAP has been identified as a potential for breast cancer therapeutics, but the specific functions of XIAP that contribute to breast cancer development or progression remain unknown. The major tasks for the first phase of this project involved establishing a human breast cancer cell line model that would be suitable for in vitro and in vivo analysis of the role of XIAP on breast cancer development and progression. Using a cell line that is amenable to both anchorage-independent growth studies in vitro and xenograft tumor formation in vivo, I substantially depleted XIAP protein expression by RNAi and subsequently re-introduced mutants of XIAP that are deficient in specific functions. In the next phase of this project, these cell lines will be evaluated to identify the role of XIAP in tumorigenesis. This knowledge will be valuable to guide the development of therapeutics that specifically target tumor-related functions of XIAP.